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Mucosally administered *Lactobacillus* surface-displayed influenza antigens (sM2 and HA2) with cholera toxin subunit A1 (CTA1) Induce broadly protective immune responses against divergent influenza subtypes

Rui Li[a,1], Mohammed Y.E. Chowdhury[a,c,1], Jae-Hoon Kim[a], Tae-Hwan Kim[a], Prabuddha Pathinayake[a], Wan-Seo Koo[a], Min-Eun Park[d], Ji-Eun Yoon[d], Jong-Bok Roh[b], Seung-Pyo Hong[b], Moon-Hee Sung[b], Jong-Soo Lee[a,3], Chul-Joong Kim[a,2,3,*]

[a] College of Veterinary Medicine (BK21 Plus Program), Chungnam National University, Daejeon, Republic of Korea
[b] BioLeaders Corporation, Daejeon, Republic of Korea
[c] Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University, Chittagong 4202, Bangladesh
[d] Foot and Mouth Disease Division, Animal Quarantine and Inspection Agency, Anyang, Republic of Korea

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ABSTRACT

The development of a universal influenza vaccine that provides broad cross protection against existing and unforeseen influenza viruses is a critical challenge. In this study, we constructed and expressed conserved sM2 and HA2 influenza antigens with cholera toxin subunit A1 (CTA1) on the surface of *Lactobacillus casei* (pgsA-CTA1sM2HA2/*L. casei*). Oral and nasal administrations of recombinant *L. casei* into mice resulted in high levels of serum immunoglobulin G (IgG) and their isotypes (IgG1 & IgG2a) as well as mucosal IgA. The mucosal administration of pgsA-CTA1sM2HA2/*L. casei* may also significantly increase the levels of sM2- or HA2-specific cell-mediated immunity because increased release of both IFN-γ and IL-4 was observed. The recombinant pgsA-CTA1sM2HA2/*L. casei* provided better protection of BALB/c mice against 10 times the 50% mouse lethal doses (MLD_{50}) of homologous A/EM/Korea/W149/06 (H5N1) or A/Aquatic bird/Korea/W81/2005 (H5N2) and heterologous A/Puerto Rico/8/34(H1N1), or A/Chicken/Korea/116/2004(H9N2) or A/Philippines/2/08(H3N2) viruses, compared with *L. casei* harboring sM2HA2 and also the protection was maintained up to seven months after administration. These results indicate that recombinant *L. casei* expressing the highly conserved sM2, HA2 of influenza and CTA1 as a mucosal adjuvant could be a potential mucosal vaccine candidate or tool to protect against divergent influenza viruses for human and animal.

1. Introduction

Influenza A viruses that cause serious respiratory illnesses belong to the family Orthomyxoviridae. Every year, approximately 3–5 million people are infected, and 250,000–500,000 individuals die worldwide due to the complications caused by this virus (Osterholm, 2005; Viboud et al., 2010). New subtypes of influenza virus are continuously evolving through the natural reassortment in the antigenic sites of the surface antigens hemagglutinin (HA) and neuraminidase in diverse hosts, such as chicken, wild birds, pigs and humans. Despite the availability of anti-influenza therapeutics, vaccinations remain a mainstay for the reduction of the substantial burden from influenza infection. However, the currently licensed inactivated virus vaccines provide protection only against specific subtypes rather than new antigenic variants and need to be updated regularly (Nichol et al., 1999). For instance, the previous seasonal influenza vaccine failed to efficiently control the spread of the H3N2 viruses in 2014–2015 in United States (D'Mello et al., 2015). Furthermore, the process required to develop an inactivated virus vaccine against potential pandemics is complicated and time consuming, usually requiring more than six months of preparation (Gerdil, 2003). Thus, the development of a rapid, productive, and effective influenza vaccine that provides

* Corresponding author at. Present address: College of Veterinary Medicine, Chungnam National University, 220 Gung-Dong, Yuseong-Gu, Daejeon 305-764, Republic of Korea.
E-mail addresses: jongsool@cnu.ac.kr (J.-S. Lee), cjkim@cnu.ac.kr (C.-J. Kim).
[1] R.L and M.Y.E.C contributed equally to this work.
[2] These corresponding authors contributed equally to this work.
[3] Jong-Soo Lee and Chul-Joong Kim contributed equally to this work.

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broad cross protection against known and unpredicted subtypes is badly needed.

The transmembrane matrix protein 2 (M2) is abundant on the surfaces of influenza virus-infected cells but rare in mature virions (Lamb et al., 1985). Previous studies have demonstrated that the extracellular domain (M2e) of M2 is well conserved across the influenza subtypes (Ito et al., 1991; Liu et al., 2005), which makes M2e an attractive target for universal vaccine development. Vaccine candidates designed with a conserved M2e are found to be protective against influenza virus infections (De Filette et al., 2006; Huleatt et al., 2008; Mozdzanowska et al., 2007). Similarly, hemagglutinin (HA) is another abundant protein on the influenza virus surface that is strongly immunogenic and constitutes a 75-kDa membrane glycoprotein in its homotrimer form (Wilson et al., 1981). During infection, viral HA binds to the sialic acid residues on host cells and enters by initiating the receptor-mediated endocytosis process (Skehel and Wiley, 2000; Wiley and Skehel, 1987; Wilson and Cox, 1990). Although HA is a promising target for designing influenza vaccines due to its immunogenicity (Stevens and Donis, 2007), the recombinant HA protein or HA DNA vaccines could induce protection only against homologous virus in mice (Lalor et al., 2008 Zheng et al., 2009). In contrast, new HA subtypes continue to emerge due to antigenic drift and shift (Carrat and Flahault, 2007; Webby et al., 2007; Wilson and Cox, 1990). Therefore, the highly conserved HA2 subunit of HA was investigated and found to be effective for cross-protective antibodies against several subtypes of viruses in mice (Bommakanti et al., 2010; Gocnik et al., 2008; Lee et al., 2013; Okuno et al., 1994; Smirnov et al., 2000). However, these M2- and HA2-based vaccines are lowly immunogenic in nature, which can result in a reduction of mortality but not morbidity in animal tests. Thus, various approaches, such as the addition of adjuvant and different administration strategies have been exploited for improving the immunogenicity of M2- and HA2-based recombinant vaccines.

Currently, mucosal vaccines are of interest due to their easy administration process. In addition, this process does not involve the risk of blood-borne infections, which may occur due to contaminated injection needles. Most importantly, a mucosal vaccine has the potential to induce both mucosal and systemic immune responses. However, only a few commercially available mucosal vaccines currently exist due to the challenges associated with the development of an effective mucosal vaccination, such as difficulties in generating effective mucosal immunity and the lack of safe, effective mucosal adjuvants and delivery systems (Ellebedy et al., 2011; Frank et al., 2012; Kiyono and Fukuyama, 2004; Rose et al., 2012; Lycke, 2012).

Lactobacillus is considered an attractive carrier that is generally regarded as safe (GRAS) and can survive in the host intestinal tract compared with others, such as *Salmonella, Shigella* and *Listeria* (Friedman et al., 2000; Lee et al., 2000; Shata and Hone, 2001; Shata et al., 2001). Previous studies have shown that specific *Lactobacillus* can induce inflammatory responses against infection, increasing specific IgA antibodies, activating the monocytic lineage (Christensen et al., 2002; Mohamadzadeh et al., 2005) and regulating the Th1 and Th2 pathways (Brisbin et al., 2010; Vintini et al., 2010). Importantly, the surface display of the antigen on *Lactobacillus* showed better effects than its intracellular display for mucosal vaccine (Lee et al., 2000). However, in addition to the mucosal delivery vehicle, various methods have been studied to optimize mucosal immunogenicity, such as the application of different adjuvants and the display of antigen on different locations (surface, cytoplasm and extracellular) (Lee et al., 2001; Robinson et al., 2004). Among the methods of vaccine development strategies, vaccine with an appropriate adjuvant via an appropriate route seems to be the best choice for eliciting antigen-specific immune responses.

Cholera toxin (CT) is well studied and is regarded as the most effective mucosal adjuvant. Antibody induced by CT can last more than two years in mice and thus potently induce long-term protection (Lycke and Holmgren, 1986; Vajdy and Lycke, 1992). However, due to the adverse effects of CT (Mutsch et al., 2004; van Ginkel et al., 2000), attempts have been made to identify nontoxic subunits with adjuvanticity through the removal of either subunit A or subunit B. Studies have shown that the toxicity can be avoided using the enzymatically active subunit A1, which cannot bind to ganglioside receptors of host cells to cause adverse reactions (Agren et al., 1998, 2000). Recent studies have revealed that cholera toxin subunit A1 (CTA1) together with antigens can also induce strong adjuvant effects similar to those induced by CT (Agren et al., 1997, 1999, 2000).

In this study, we designed a consensus sM2 without its transmembrane domain and found that it was able to induce protection against divergent influenza subtypes, as indicated in our previous report (Chowdhury et al., 2014a). To improve the protection level and reduce the morbidity, we selected the HA2 gene from H5N1 as another component of the vaccine. Additionally, to induce a strong mucosal immune response, we applied CTA1 as an adjuvant and *Lactobacillus casei* as a delivery vehicle. The well-studied transmembrane protein pgsA is able to fuse the target protein to its C terminus and stabilize the complex by anchoring it to the cell membrane (Lee et al., 2006), indicating that it can be used as an anchor protein. We constructed and expressed the fusion constructs pgsA-sM2HA2 and pgsA-CTA1sM2HA2 on the surface of *L. casei*, and demonstrated that mucosal administration with recombinant *L. casei* induced systemic and mucosal immune responses that have the potential to protect against the lethal challenges of divergent influenza subtypes in a murine model.

2. Materials and methods

2.1. Animals, immunization, virus challenge and sample collection

Female BALB/c mice (approximately 5 weeks of age) were purchased from Samtako (Seoul, Korea) and housed in ventilated cages. The mice were fed sufficient commercial feed and tap water in a specific-pathogen-free environment following permission from the Institutional Animal Care and Use Committee of Bioleaders Corporation, Daejeon, Republic of Korea under protocol number BSL-ABLS-13-012. The mice were immunized in biosafety level 2 laboratory facilities. All efforts were made to minimize suffering following immunization and challenge.

The mice were divided into six experimental sets, each of which consisted of two subsets (one for oral and one for intranasal administration) with four groups each. Of the six sets, four sets had 14 mice per group (three for mucosal IgA, five for survival and six for lung virus titer at three and five day post-infection)). One set had 17 mice per group (an additional three mice for CTL at one week after vaccination), and the last set contained 11 mice per group (three for mucosal IgA, three for CTL and lymphocyte proliferation assay and five for long lasting survival). The mice were vaccinated with recombinant *L. casei* harboring the pKV-pgsA-sM2HA2 or pKV-pgsA-CTA1sM2HA2 plasmid either orally via intragastric lavage on days 1–2, 7–9 and 21–23 or intranasally (i.n.) via pipette drop to the nostrils of the anesthetized mice on days 1–2, 7–9 and 21. The doses administered through oral and intranasal inoculation were 10^{10} and 10^{9} colony forming units (CFUs) of recombinant *L. casei* in 100 and 20 μl of PBS per mouse, respectively. The control mice in the oral and intranasal groups were given *L. casei* harboring Pkv-pgsA or PBS.

Five different subtypes of avian influenza viruses, namely A/EM/Korea/W149/06 (H5N1), A/Puerto Rico/8/34(H1N1), A/Aquatic bird/Korea/W81/2005(H5N2), A/Philippines/2/08(H3N2) and